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Flower color modifications of *Torenia hybrida* by cosuppression of anthocyanin biosynthesis genes

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Abstract

White and blue/white varieties of *Torenia hybrida* were successfully obtained from the blue variety cv. Summerwave (SWB) by cosuppressing expression of two of the genes involved in anthocyanin biosynthesis; chalcone synthase (CHS) and dihydroflavonol 4-reductase (DFR). Such molecular breeding is the only precise and efficient way to create flower color variation in SWB due to its male and female sterility. Flower color and the degree of suppression varied between transgenic lines, and anthocyanin biosynthesis was more consistently suppressed in the dorsal petal lobes, ventral petal lobes and corolla tube than lateral petal lobes. A pink variety was obtained by cosuppressing the flavonoid 3′,5′-hydroxylase (F3′5′H) gene. Yellow torenia was obtained from T-33, an in-house cultivar that contained both carotenoids and anthocyanins, by cosuppression of CHS or DFR genes.

Introduction

Molecular breeding is a powerful method of plant breeding because it is possible to change a specific characteristic of a plant without sacrificing other desirable characteristics. From an aesthetic point of view, flower color is the most important characteristic in floricultural crops. Color is predominantly influenced by two types of pigments; flavonoids and carotenoids. Plant carotenoids are red, orange and yellow lipid-soluble pigments found embedded in the membranes of chloroplasts and chromoplasts [2]. Anthocyanins are a colored class of flavonoids that are responsible for the pink, red, violet and blue colors of flowers. Anthocyanins are water-soluble compounds that accumulate in vacuoles or anthocyanoplasts. The anthocyanin biosynthesis pathways of many plants, summarized in Figure 1, have been well established [7, 13, 16].

Chalcone synthase (CHS) and dihydroflavonol 4-reductase (DFR) are the first specific enzymes in flavonoid and anthocyanin biosynthesis, respectively. The genes encoding the two enzymes have been isolated from many kinds of plants [9]. The presence of flavonoid 3′,5′-hydroxylase (F3′5′H), a cytochrome P-450 [12], is almost always essential for the production of blue to purple anthocyanins (Figure 1). Flower color is generally reddish in the absence of this gene.

Torenia (*Torenia fournieri*), belonging to the Scrophulariaceae, is one of the most commercially important bedding plants. Suntory Ltd. has developed a new type of torenia cultivar Summerwave (*T. hybrida*), which has many characteristics superior to common torenia cultivars. Summerwave originally has blue flowers. Because its male and female sterility predicted the difficulty of conventional breeding a molecular approach was applied to widen flower color variation.

Gene silencing is a common phenomenon in transgenic plants and it affects transgenes and endogenous genes [22]. Petunia, and its flavonoid genes, have been providing an appropriate model system to study post-transcriptional gene silencing. Significant biochemical, biological and genetic knowledge has been

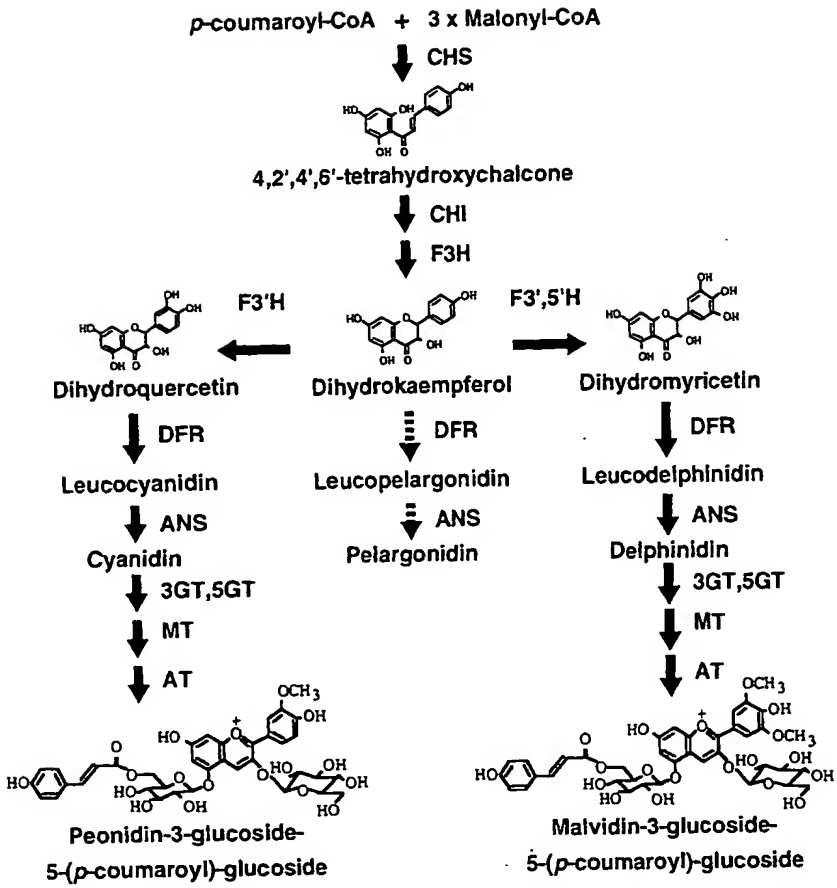

Figure 1. Schematic representation of the biosynthesis pathway for anthocyanin pigment production in *Torenia hybrida* cv. Summerwave Blue. The reactions shown by dotted arrows do not naturally occur in SWB. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose:flavonoid-3-*O*-glucosyltransferase; 5GT, UDP-glucose:flavonoid-5-*O*-glucosyltransferase; MT, anthocyanin *O*-methyltransferase; AT, anthocyanin acyltransferase.

accumulated and cell-specific gene silencing has been easily, closely and clearly observed. Both constitutive expressions of a sense [18, 25] and antisense [24] petunia CHS gene in transgenic petunia results in an altered flower pigmentation. The flower color patterns and stability of cosuppression varies significantly among inter- and intra-transgenics. Although suppression of anthocyanin biosynthesis has been reported in some species [4, 6, 10], none of the new lines appear to have commercial value. In this report, we describe transgenic torenia plants harboring sense cDNAs encoding CHS, DFR and F3′5′H enzymes isolated from torenia Summerwave Blue (SWB).

Materials and methods

Plant material and genetic transformation

Torenia hybrida cv. Summerwave Blue (Suntory, Japan) and *T. hybrida* inbred line T-33 were grown under standard greenhouse conditions. *In vitro* cultured shoots used for transformation were maintained on a MS agar medium [23]. SWB and T-33 were transformed using the method of *Agrobacterium tumefaciens* mediated transformation of *T. fournieri* [1]. Only a single shoot from each explant was rooted and further characterized.

Pigment analysis

Reflectance of petals was quantified using a spectrophotometer CM-2022 (Minolta, Japan). Extraction,

HPLC analysis and structural determination of anthocyanins were described previously [8].

Molecular analysis

Most of the molecular analysis techniques have been described previously [3, 20]. A cDNA library constructed in the UNI-ZAP (Stratagene) was screened with *Perilla frutescence* CHS (AB002582), DFR (AB002817) and gentian F3′5′H (D85184) to obtain cDNAs were.

Construction of binary vectors

The 1.4 kb *Bam*HI-*Xho*I fragment of pTCH6 containing the whole CHS cDNA, the 1.1 kb *Bam*HI-*Xho*I fragment of pTDF10 containing a partial DFR cDNA and the 0.9 kb *Bam*HI-*Xho*I fragment of pTHF2 containing a partial F3′5′H cDNA were inserted in the sense orientation between the enhanced CaMV 35S promoter and the nopaline synthase terminator of pBE2113-GUS [15]. pBE2113-GUS has nptII as the selection marker. The resultant plasmids were designated pBETC6, pBETD10 and pBETF10, respectively. Binary vector pKIWI105 was used as a positive control for transformation and gene expression.

Results

Anthocyanin biosynthesis pathway in Torenia

Over 80% of the total anthocyanins in the petals of SWB was malvidin 3-*O*-*β*-D-glucoside-5-*O*-(6-*O*-*p*-coumaroyl)-*β*-D-glucoside. The petals also contained malvidin 3,5-diglucoside and peonidin derivatives as minor anthocyanins. Torenia CHS (pTCH6, AB012923), DFR (pTDF10, AB012924) and F3′5′H (pTHF2, AB012925) cDNAs were isolated and sequenced. They showed good homology to their counterparts from other species. In particular torenia CHS and DFR amino acid sequences showed high homology (90% and 71%, respectively) to those of snapdragon, which also belongs to the family Scrophulariaceae. It is known that the pathways leading to anthocyanidins 3-glucoside are well conserved [13]. The anthocyanin biosynthesis pathway of torenia was deduced as shown in Figure 1.

Transgenic plant phenotypes obtained with CHS and DFR cosuppression

Torenia SWB was transformed with the binary vector pBETC10 containing full-length torenia CHS sense cDNA. The binary vector pBETD6 with sense direction also use to transform SWB. pBETD6 contained a partial torenia DFR cDNA lacking 300 bp of the 5′ region because a restriction enzyme site in the coding region was used in plasmid construction. These cDNAs are under the control of the enhanced CaMV 35S promoter [15]. Therefore, transcripts from the DFR transgene can not be translated, because of absence of functional open reading frames.

Many of the transgenic plants had petals with reduced amounts of anthocyanins. Both transgenic plants of SWB CHS (Figure 2a) and SWB DFR (Figure 2d) showed the same phenotypic pattern. The degree of the reduction varied with each transgenic line, and the petals had a range of colors from blue (Figure 2a left), to blue and white (Figure 2a center), and pure white (Figure 2a right). Interestingly, anthocyanin production was more consistently suppressed in the dorsal and ventral petal lobes, and the corolla tube, than in the lateral petal lobes.

The degree of suppression of petal colors was classified into six groups, shown in A–F of Figure 2d. Phenotype A had a small region of pale blue or white color specifically in the inside of the dorsal and ventral petal lobes. In phenotype B, a white color region was larger in the dorsal and ventral petal lobes, and the corolla tube was colored pale blue. Phenotype C had a blue color in the lateral petal lobes and was almost completely white in other parts, except for a little pale blue color in the corolla tube. In phenotype D, only lateral petal lobes showed blue color. Phenotype E had a white color in all regions, with the exception of the tip of the lateral petal lobes. Phenotype F had a pure white color. Of the 121 SWB CHS transgenic plants studied, thirty plants (ca. 25%) showed phenotypic changes in flower color (Table 1). Pure white-colored petals (phenotype F) were observed in over 60% of the plants with altered flower color. No petal color changes were observed in the plants transformed with pKIWI105.

Although SWB DFR plants showed a higher frequency (45%) of flower color change than SWB CHS plants, color changes were equally distributed across phenotypes A–F. One line of SWB plants transformed with pTDF10, SWB/DFR 13-66, had white color only in the corolla tube (Figure 2j). SWB/DFR 13-7 seemed unstable, indicated by unchanged pigment at

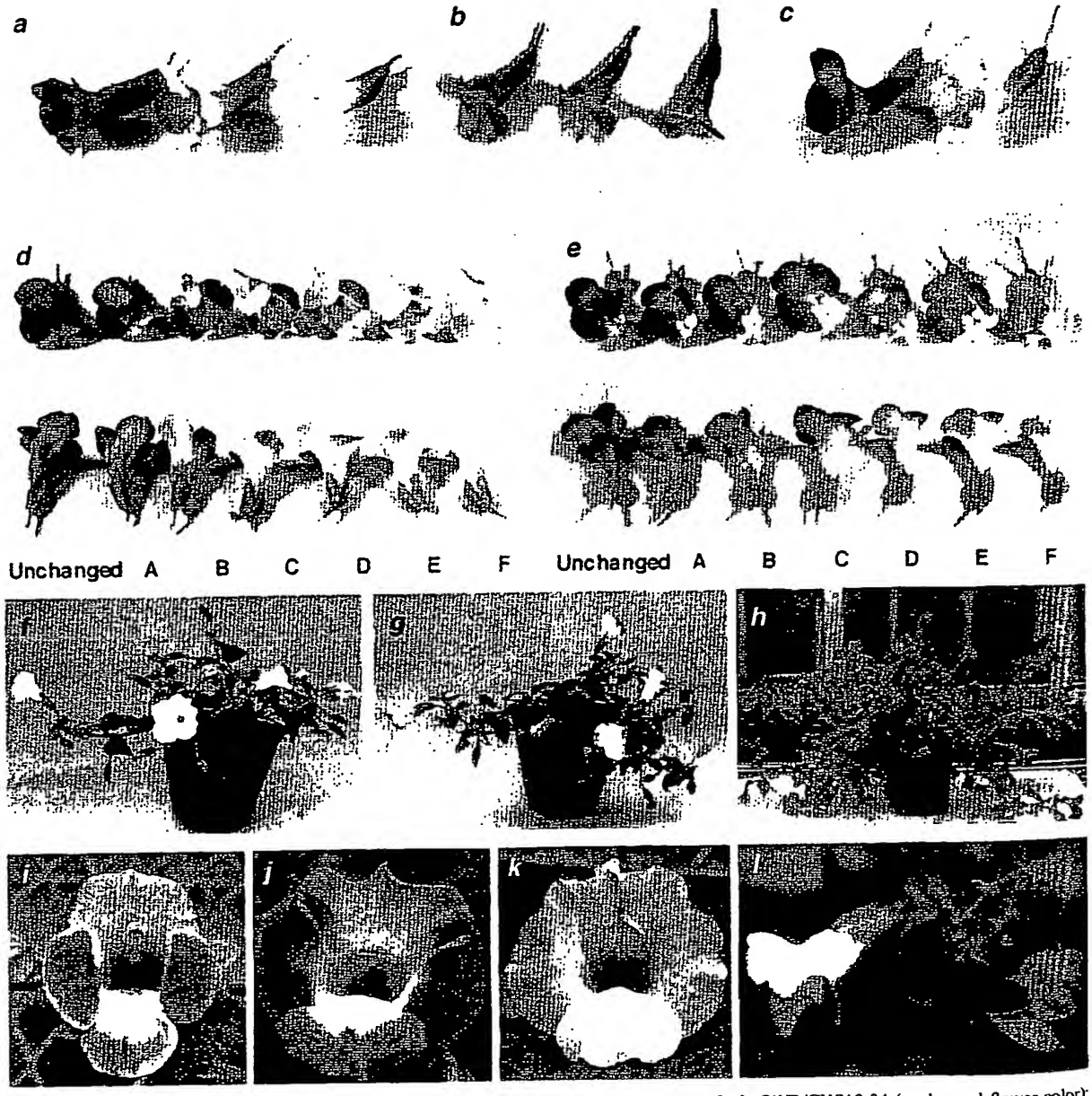

Figure 2. Flower phenotype of CHS sense, DFR sense and F3′5′H sense transformants. a. Left: SWB/CHS10-34 (unchanged flower color); center: SWB/CHS 11-1 (only blue pigmentation in lateral petal lobes and white in another parts, phenotype C); right: SWB/CHS 11-65 (pure white, phenotype F). b. Left: SWB (untransformed flower); center: SWB/F3′5′H 20-26 (pink in corolla tube and purple in petal, phenotype D); right: SWB/F3′5′H 20-68 (pink petal without color in dorsal (upper or adaxial) lobes, phenotype F). c. Left: T33/CHS 10-99 (unchanged); right: T33/CHS 10-38 (pale yellow carotenoid pigments, phenotype F). d, e. Flower of SWB (d) and T-33 (e) transgenic plants harboring partial DFR sense cDNA. Shown from left to right: unchanged flower color, phenotype A to F, respectively. Flower pigmentation was absent in the dorsal petal lobes, ventral petal lobes and corolla tube (f–h). f. Pure white (SWB/DFR 13-20). g. Blue and white (SWB/CHS 11-64). h. Untransformed SWB. i. Pink flower of SWB/F3′5′H 20-68. j, k. Irregular phenotype. White only in corolla tube: SWB/DFR 13-66 (i) and pale blue in whole corolla: SWB/F3′5′H 20-68 (k). l. Novel phenotypes in the same branch of SWB/DFR 13-7. Blue and white flower at fourth flower (left) and blue flower at fifth flower (right). The blue flower is very similar to control, and could be a revertant.

Table 1. Frequency of flower color change in transgenic plants. UC, unchanged flower color; IC, irregularly changed flower color; A to F, phenotypes A to F described and shown in Figure 2.

Host plant	Transformed gene	Number (%) of transgenic plants								
		Total	UC	IC	A	B	C	D	E	F
SWB	CHS	121	91	0	4	0	3	0	4	19
			(75%)	(0%)	(3%)	(0%)	(2%)	(0%)	(3%)	(16%)
SWB	DFR	115	63	1	9	4	8	12	10	8
			(55%)	(1%)	(8%)	(3%)	(7%)	(10%)	(9%)	(7%)
T33	CHS	96	64	0	10	9	10	2	0	1
			(67%)	(0%)	(10%)	(9%)	(10%)	(2%)	(0%)	(1%)
T33	DFR	80	46	0	12	4	9	3	4	2
			(58%)	(0%)	(15%)	(5%)	(11%)	(4%)	(5%)	(3%)
SWB	F3′5′H	105	88	1	0	3	0	9	0	4
			(84%)	(1%)	(0%)	(3%)	(0%)	(9%)	(0%)	(4%)

Table 2. Amount of anthocyanidin production in petals of transgenic plants. The reflectance is quantified to the whiteness of the petals such that pure blackness shows 0% reflectance and pure white 100% reflectance). Pel, pelargonidin; Cya, cyanidin type-anthocyanidins; Del, delphinidin type-anthocyanidins.

Flower color	Transgenic line line	Reflectance (%)	Anthocyanidin (mg/g petal)			
			Pel	Cya	Del	total
Blue	SWB/KIWI 1-1-1	1.1	0	0.178	1.349	1.527
Pale blue	SWB/CHS 13-37	1.8	0	0.074	0.636	0.710
	SWB/DFR 11-18	4.2	0	0.092	0.616	0.707
Blue and white	SWB/CHS 14-8	4.8	0	0.042	0.596	0.637
	SWB/DFR 11-1	12.4	0	0.066	0.402	0.468
White	SWB/CHS 13-20	55.2	0	0	0.043	0.043
	SWB/CHS 10-20	61.5	0	0.009	0.041	0.050
Pink	SWB/F3′5′H	9.7	0.013	0.410	0	0.423

the fifth flower position (Figure 2l). The observation in SWB/DFR 13-7 is similar to the revertant branch reported in the progeny of a petunia CHS transgenic plant [22]. The revertant branch was reported to be caused by deletion of the inverted repeat transgene. Several lines selected from phenotype F (pure white, Figure 2f) and from phenotype C and D (blue and white, Figure 2g) were examined and proven to be stable after vegetative propagation in one year in a greenhouse. Results of southern hybridization showed that 1 or 2 copies of the transgene were introduced in selected lines (data not shown).

Northern and pigment analyses of CHS/DFR transgenics

The reduction of expression of CHS and DFR mRNA and the amount of anthocyanidin in the transgenics were correlated. For detailed analysis, several transgenics of SWB were selected from each of the phenotypes A-B (SWB/CHS 11-18, 11-20 and SWB/DFR 13-5, 13-37), C-D (SWB/CHS 11-1, 11-55 and SWB/DFR 13-18, 13-19, 14-8), and E-F (SWB/CHS 10-20, 11-9, 11-57 and SWB/DFR 13-8, 13-20, 13-52). It was confirmed that the reflectance increased as the amount of anthocyanidins decreased (Table 2) and that the reduction of anthocyanin concentration was correlated to change in flower color. In order to study the correlation between gene suppression and anthocyanin accumulation, CHS and DFR transcripts

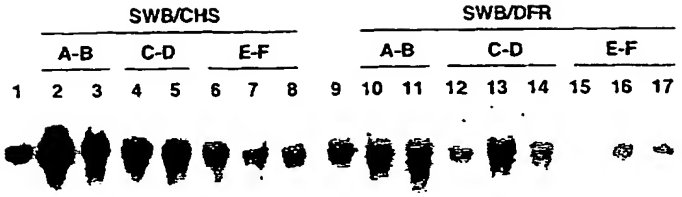

Figure 3. CHS and DFR floral mRNA expression in SWB transgenic plants determined by Northern blot analysis. RNA analysis of total RNA (10 μg) isolated from young petals of opening flowers was performed. Phenotype A and B, C and D, E and F were grouped, respectively. 1 and 9, SWB/KIWI 1-1-1; 2, SWB/CHS 11-18; 3, SWB/CHS 11-20; 4, SWB/CHS 11-1; 5, SWB/CHS 11-55; 6, SWB/CHS 10-20; 7, SWB/CHS 11-9; 8, SWB/CHS 11-57; 10, SWB/DFR 13-5; 11, SWB/DFR 13-37; 12, SWB/DFR 13-18; 13, SWB/DFR 13-19; 14, SWB/DFR 14-8; 15, SWB/DFR 13-8; 16, SWB/DFR 13-20; 17, SWB/DFR 13-52. CHS and DFR mRNA were detected with ^{32}P-labeled full-length CHS and DFR cDNA as a probe, respectively.

in the petals were analyzed. Petals in phenotype A-B (SWB/CHS 11-18) had more mRNA than the control line SWB/KIWI 1-1-1 expressing GUS protein (Figure 3). mRNA level was lower in phenotypes C-F and SWB/DFR 13-8 rarely showed any signal. Anthocyanins accumulate only in the epidermal cell layer of the SWB petal (data not shown). Many genes of the flavonoid biosynthesis enzyme are expressed specifically in this cell layer [11]. The transgene driven by the enhanced CaMV 35S promoter is likely to be expressed in all cell layers of the petals and most of the message detected in our results may have been derived from the transgene.

Phenotype of plants transformed with F3′5′H cDNA

The binary vector pBETF10 containing the partial torenia F3′5′H cDNA containing only about 600 bp of the 3′ region in a sense direction with respect to the promoter was also used to transform SWB. Four out of 105 transgenic plants changed flower color from blue to pink (Figure 2b). This color change was consistently observed to be stronger in the dorsal petal lobes, ventral petal lobes and the corolla tube than in the lateral petal lobes. This same distribution was also observed in SWB/CHS and SWB/DFR plants. Transgenic lines showing intermediate phenotypes had pink colored flowers only in the corolla tube (Figure 2b center). Anthocyanidins in completely pink flowers were analyzed (Table 2) and no anthocyanins derived from delphinidin were detected. An increased amount of cyanidin and peonidin compared with controls and a small amount of pelargonidin were observed. The suppression of the F3′5′H gene therefore caused a diversion from the delphinidin pathway to the cyanidin pathway (Figure 1). The dorsal petal lobes of the pink flowers were white (Figure 2i) which could be due to a lack of F3′H gene expression in this region. One irregularly changed flower color line was observed; SWB/F3′5′H 10-77 showed pale blue in all parts of the corolla (Figure 2k).

Transformation of T-33

Torenia T-33 petals, which contain both anthocyanins and carotenoids, are dark brown and purple (Figure 2c left). T-33 was transformed with the binary vectors pBETC10 and pBETD6 using the same methods as with SWB. About 40% of the transgenic plants changed flower color (Table 1). The patterns of anthocyanin reduction in each construct were observed to be the same as SWB/CHS and SWB/DFR (Figure 2c and 2e), except for the presence of pale yellow lines (phenotype F, Figure 2c right) (Table 1). Yellow transgenic torenia was therefore successfully generated by specifically suppressing the anthocyanin biosynthetic pathway, without obviously affecting carotenoid biosynthesis.

Discussion

White flower lines have been obtained from purple petunia and pink chrysanthemum through cosuppression of the CHS gene [4]. The same strategy has produced pink flowers from red rose and red carnation [10]. These flowers have not been commercialized, probably due to their limited market value. In contrast Summerwave was awarded the best cultivar at PLANTECH 1995 and has high marketability. However, it can not be further bred using a cross-hybridization method because both male and female gametophyte developments of SWB are incomplete (T. Higashiyama and T. Kuroiwa, pers. comm.). Although mutational breeding is a good tool for gene

disruption, we have already screened several thousand SWB mutation lines from somaclonal variation and heavy-ion beam irradiation without finding a line with pure white flowers (S. Tsuda and K. Suzuki, unpubl.). Not only was the molecular breeding for flower color in Summerwave successful, but its morphological habits were retained. Common torenia cultivars have compact standing branches as a small bushy plant, whereas Summerwave has widely spread cascading branches (Figure 2h). White (Figure 2f) and blue/white (Figure 2g) transgenic plants retained such characteristics.

Flower color has been a useful characteristic for monitoring gene suppression by both sense and antisense suppression. Post-transcription gene silencing of the CHS gene in petunia results in pure white flowers, or flowers with a variegated pigmentation phenotype. The mechanism of the phenomena has been studied in detail [14, 18, 22, 23, 25]. Jorgensen *et al.* reported that 63% and 11% of the 185 sense CHS transgenic plants studied had corollas with altered colors and white flowers, respectively [14]. SWB CHS transgenic plants showed phenotypic change with a frequency similar to petunia (Table 1) though variegated pigmentation was not observed in SWB. In chrysanthemum [4], rose and carnation [10], the frequency of CHS suppression with sense CHS gene was very low and no variegated phenotypes were observed. Therefore, cosuppression phenomena may be different among plant species. Although the molecular mechanism of post-transcriptional gene silencing has been discussed, the mechanism and its biological significance have not been fully understood [5]. Further molecular analysis of these torenia plants, showing new and simple patterns of sense suppression, should be helpful for a better understanding of post-transcriptional gene silencing. Transgenic lisianthus with antisense suppression of CHS gene showed several novel flower color patterns including that similar to our results [6]. The simple patterns observed in SWB may be explained by the hypothesis for the mechanism of cosuppression that small complementary RNAs (cRNAs) could traffic or diffuse into adjacent cells, initiate turnover of homologous sequences, and then cause the production of new cRNAs as a wave of cosuppression [19]. DFR may be a better choice than CHS because DFR suppression does not suppress flavonol and flavone synthesis which are both important in the UV protection of plants [21].

Transgenic T-33 lines harboring CHS or DFR genes also had reduced anthocyanin pigments but with a lower frequency than was observed for SWB. This might be because the torenia CHS and DFR genes were isolated from SWB. The nature of the sense transgene construct is likely to be important for cosuppression [14].

Two lines of irregular phenotypes were observed possibly as a result of T-DNA insertion or somaclonal mutation (Figure 2k).

Flowers of *Torenia hybrida* cv. Summerwave predominantly accumulate malvidin, a delphinidin derivative. Cosuppression of F3′5′H successfully yielded pink flowered plants which contain mainly peonidin, indicating the critical role of F3′5′H in producing blue flowers. We also showed that anthocyanin pathway could be specifically suppressed without affecting the carotenoid biosynthesis pathway, showing the usefulness of molecular breeding to change a target character whilst preserving other characteristics. This strategy should be applied to generate yellow flowers from other species containing both anthocyanins and carotenoids. Further molecular breeding to widen the range of flower color of SWB is in progress.

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Flower color modulations of *Torenia hybrida* by downregulation of chalcone synthase genes with RNA interference

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Abstract

Suppression of biosynthetic genes involved in flower color formation is an important approach for obtaining target flower colors. Here we report that flower color of the garden plant *Torenia hybrida* was successfully modulated by RNA interference (RNAi) against a gene of chalcone synthase (CHS), a key enzyme for anthocyanin and flavonoid biosynthesis. By using each of the coding region and the 3′-untranslated region of the CHS mRNA as an RNAi target, exhaustive and gene-specific gene silencing were successfully induced, and the original blue flower color was modulated to white and pale colors, respectively. Our results indicate that RNAi is quite useful for modulations of flower colors of commercially important garden plants.

Keywords: RNAi; *Torenia hybrida*; Chalcone synthase; Flower color

1. Introduction

Artificial modulations of flower colors are one of the important subjects of current plant biotechnology. Anthocyanins, a member of flavonoids, substantially contribute to flower color formation (Buchanan et al., 2000). Therefore, artificial regulation of anthocyanin biosynthesis can be a useful approach for obtaining flowers of desired colors. Actually, several attempts to change flower colors have been performed by down-regulating anthocyanin biosynthetic genes with antisense or co-suppression technique (Forkmann and Martens, 2001).

However, recent studies evidently demonstrated that double-stranded RNA (dsRNA)-mediated gene silencing phenomenon called 'RNA interference (RNAi)' is more potent than the above two techniques to suppress gene functions. During the process of RNAi, long dsRNA is cleaved into ~21 nt small interfering RNAs (siRNAs), and the siRNAs then guide sequence-specific mRNA degradation (Denli and

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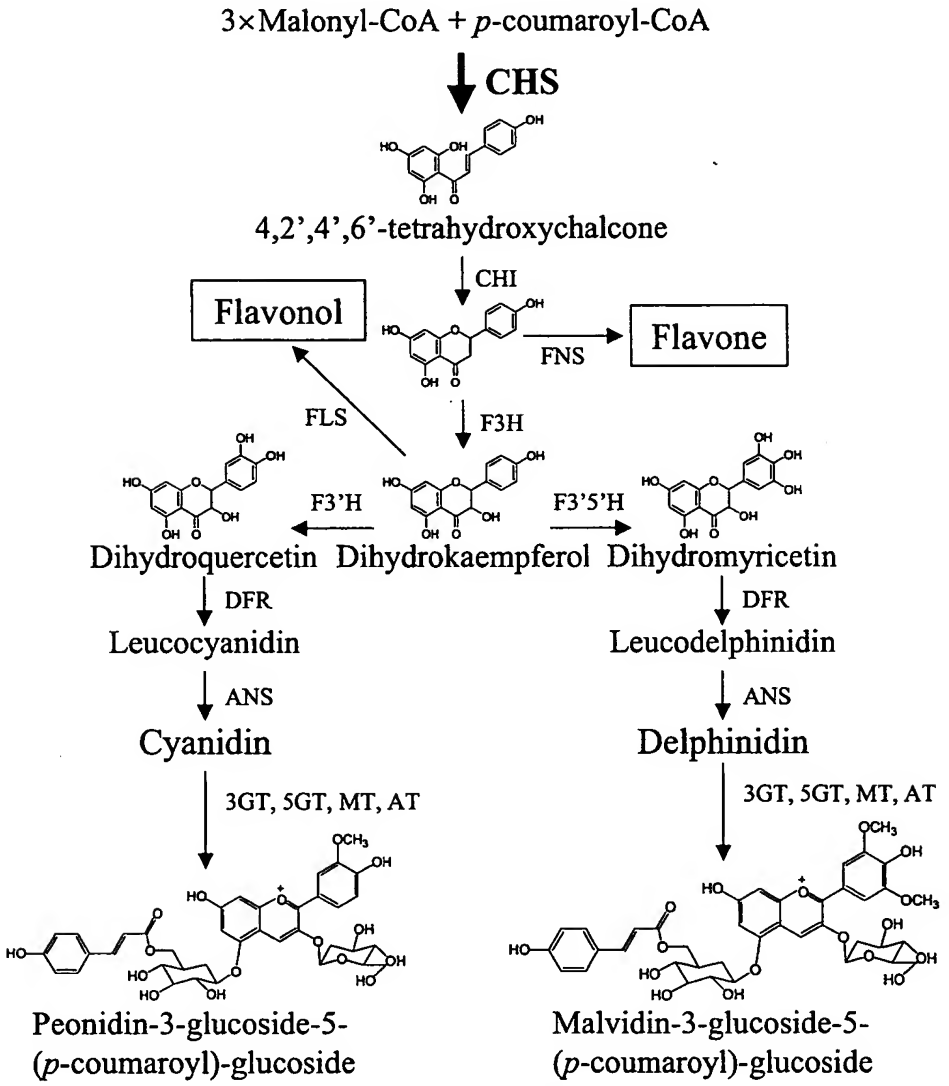

Fig. 1. Biosynthetic pathway of anthocyanins in *T. hybrida* (Suzuki et al., 2000). CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose:flavonoid-3-*O*-glucosyltransferase; 5GT, UDP-glucose:flavonoid-5-*O*-glucosyltransferase; MT, anthocyanin *O*-methyltransferase; AT, anthocyanin acyltransferase.

Hannon, 2003). Since the discovery in the nematode *Caenorhabditis elegans* (Fire et al., 1998), RNAi has been used as a powerful tool for functional genomics in many eukaryotes, including plants.

By using the RNAi technique, we attempted to modulate flower colors of *Torenia hybrida* cv. Summerwave Blue, a commercially important garden plant, by downregulating a chalcone synthase (CHS) gene. CHS is the first committed enzyme of anthocyanin and flavonoid biosynthesis (Fig. 1). To date, flower color modulations of *Torenia* have been performed by suppressing gene expression of CHS or dihydroflavonol 4-reductase (DFR: Fig. 1) by antisense and co-suppression techniques (Aida et al., 2000; Suzuki et al., 2000). Here we report that flower color of *T. hybrida* was successfully modulated by RNAi technique. By using distinct mRNA sites (the coding region and the 3'-untranslated region) as RNAi

targets, different flower colors were obtained. Our results clearly demonstrate that RNAi is quite useful for modulations of flower colors.

2. Materials and methods

2.1. Plasmid construction

Full-length cDNA of the *T. hybrida* CHS (Suzuki et al., 2000; designated as *ThCHS1* in this paper; DDBJ accession no. AB012923) was obtained from buds by RT-PCR using the forward primer (5′-GCGGTTGCCTCTCTGATTAT-3′) and the reverse primer (5′-CTTCACAATCACGACACACG-3′), and cloned into pCR2.1 (Invitrogen, USA) to produce pCR2.1-CHS. A binary vector (CDSi vector) including an inverted repeat sequence of a part of the coding region (CDS) of *ThCHS1* (black arrow in Fig. 2a; 610 bp in length) was constructed as follows. A part of the CDS of *ThCHS1* (610 bp: position 304–913) was amplified with two primers, CDS-f (CGAGCTCAGAAGCGTTACATGCACCTC) and CDS-r (CGGGATCCTCTTTCAGGAGGTGGAAGGT) using pCR2.1-CHS as a template, and cloned into pCR2.1 (Invitrogen, USA) to produce pCR2.1-CDS. pCR2.1-CDS was digested with *Bam*HI, and then blunt-ended with T4 DNA polymerase to destroy *Bam*HI site, and self-ligated. The plasmid (pCR2.1-CDS with no *Bam*HI site) was digested with *Sac*I, and then ligated to the *Sac*I treated fragment of pBI121 (Jefferson et al., 1987) to produce pBI121-CDS. pCR2.1-CDS was digested with *Bam*HI and *Xba*I, and then ligated to *Bam*HI–*Xba*I fragment of pBI121-CDS to produce CDSi vector, in which β-glucronidase (GUS) gene region originally included in pBI121 was used as a spacer (loop) sequence. A binary vector (3′-UTRi vector) including an inverted repeat sequence of a part of the 3′-untranslated region (3′-UTR) of *ThCHS1* (gray arrow in Fig. 2a; 132 bp in length) (Fig. 2b) was constructed as follows. A part of *ThCHS1* gene including a part of the CDS and the 3′-untranslated region (3′-UTR; position 840–1406) was amplified with two primers,

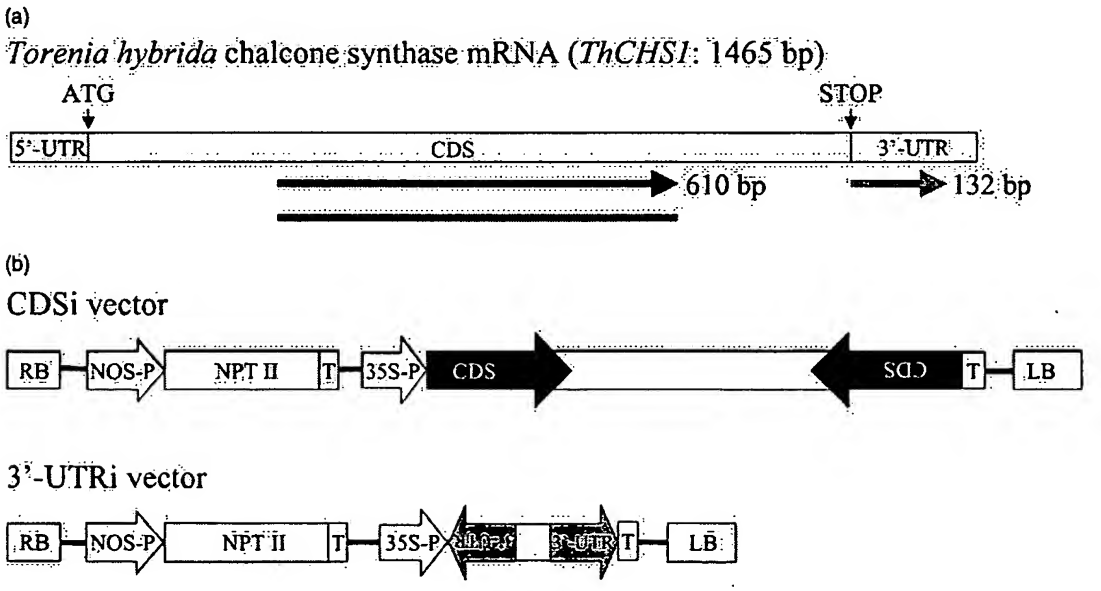

Fig. 2. Schematic representation of the structures of the *T. hybrida* CHS gene (*ThCHS1*) and RNAi vectors. (a) Schematic diagram of the structure of *T. hybrida* CHS mRNA. ATG, start codon; STOP, stop codon; 5′-UTR, 5′-untranslated region; CDS, coding region, 3′-UTR, 3′-untranslated region; black arrow, a region used for CDSi vector; gray arrow, a region used for 3′-UTRi vector; black line: a region used for Northern and Southern analysis as a probe. (b) Schematic diagrams of the structures of RNAi vectors. Inverted repeat was indicated by black (encoding a part of the CDS) or gray (encoding a part of the 3′-UTR) arrow. RB, right border; NOS-P, promoter sequence from nopaline synthase gene; NPT II, neomycin phosphotransferase; T, terminator sequence from nopaline synthase gene; 35S-P, CaMV 35S promoter; CDS, coding region; LB, left border; 3′-UTR, 3′-untranslated region.

3′-UTR-f1 (CGGGATCCATACTACCGGACAGCGAAG) and 3′-UTR-r1 (CGAGCTCCTTCACAATCACGACACACG) using pCR2.1-CHS as a template, and digested with *Bam*HI and *Sac*I simultaneously. The digested fragment was then ligated to the corresponding fragment of pUC18 to produce pUC-UTR1. Another part of the *ThCHS1* gene including a part of the 3′-UTR (132 bp: position 1275–1406) was amplified with two primers, 3′-UTR-f2 (CGGGATCCTGGATTTGATGTTTTAATG) and 3′-UTR-r2 (GCTCTAGACTTCACAATCACGACA) using pCR2.1-CHS as a template, and then digested with *Bam*HI and *Xba*I simultaneously. The digested fragment was next ligated to the corresponding fragment of pUC18 to produce pUC-UTR2. pUC-UTR1 was digested with *Bam*HI and *Sac*I simultaneously, and ligated to the corresponding fragment of pUC-UTR2 to produce pUC-UTRi. pUC-UTRi was double-digested with *Sac*I and *Xba*I, and then ligated to *Sac*I–*Xba*I fragment of pBI121 to produce 3′-UTRi vector, in which the last 435 bp of the CDS (position 840–1274) were used as a spacer (loop) sequence.

2.2. Plant materials and transformation

Torenia hybrida cv. Summerwave Blue (Suntory Ltd., Japan) was obtained from a commercial plant distributor. *T. hybrida* plants were grown and transformed as described previously (Suzuki et al., 2000). Insertion of the RNAi construct into the genome was confirmed by genomic PCR using the following primers. Primers for CDSi were: 5′-AGCGTAATGCTCTACACCACG-3′ (forward primer) and 5′-TGGCAATACTCCACATCACC-3′ (reverse primer). Primers for 3′-UTRi were: 5′-ATACTACCGGACAGCGA-3′ (forward primer) and 5′-TCTTTCAGGAGGTGGAAGGT-3′ (reverse primer).

2.3. Anthocyanin analysis

Fresh petals (1 g fresh weight) were soaked in 1 ml of extraction solvent (0.5% (v/v) trifluoroacetic acid (TFA) in 50% acetonitrile) for 20 min at room temperature. After centrifugation at 20,000 × *g* for 2 min at room temperature, supernatants were collected, and applied to water-equilibrated Sep-Pak Plus C-18 Cartridges (Waters, USA). Anthocyanins were eluted with 3 ml of the extraction solvent, and lyophilized. The lyophilized samples were then dissolved in 500 μl of the extraction solvent, and used for HPLC analysis. Samples (10 μl) were applied to Mightsil RP-18 GP250-4.6 (5 mm) column (Kanto Kagaku, Japan) and eluted with a linear gradient of 0–60% of solvent A (0.5% TFA in acetonitrile) in solvent B (0.5% TFA in water) at a flow rate of 0.6 ml/min over 30 min. The column temperature was 40 °C and effluent was monitored at 530 nm. The absolute quantities of anthocyanins in 1 g of petals were determined from peak areas of chromatograms against a standard curve. The standard curve was obtained with peonidin-3-glucoside-5-(*p*-coumaroyl)-glucoside purified from WT flowers independently as an authentic sample. In addition to the quantitative analysis, qualitative analysis was carried out by scanning of UV-Vis absorption profiles of effluent with photo-diode array (PDA) in the range of 200–600 nm.

2.4. Northern and Southern analysis

Total RNA was extracted from buds 1–7 days before flowering. Three or four buds were ground in liquid nitrogen with a mortar and pestle. The powder was transferred to a tube containing 600 μl of extraction buffer (4.2 M guanidine isothiocyanate, 0.5% sarkosyl, 25 mM sodium citrate) and mixed. After centrifugation at 12,000 × *g* for 5 min at room temperature, supernatant was transferred to a new tube and extracted twice with equal volumes of phenol–chloroform. RNA was precipitated with ethanol, and dissolved in 400 μl of nuclease-free water. RNA was reprecipitated with LiCl, and dissolved in 50 μl of nuclease-free water. Total RNA (8 μg) was separated on 1.5% agarose gels containing 2.2 M formaldehyde, and transferred to a nylon membrane (Hybond-N^+; Amersham Biosciences, UK). PCR product encoding a part of the CDS (610 bp; black line in Fig. 2a) was used as a probe. Hybridization was performed at 42 °C in 50% formamide, 0.12 M Church phosphate buffer (Church and Gilbert, 1984), 0.25 M NaCl, 7% SDS, 1 mM EDTA using a probe labeled by random priming in the presence of (α-^{32}P) dCTP. Blots were washed with a wash buffer (0.1 × SSC, 0.1% SDS) at 68 °C. Radioactivity was detected using an X-ray film. Genomic DNA was isolated as described previously (Aida et al., 2000). DNA (10 μg) digested with *Hin*dIII or *Eco*RI was separated on 1% agarose gels and transferred to

Hybond-N^+. Subsequent procedures were identical to Northern analysis except that hybridization buffer did not contain formamide, and hybridization and washing were carried out at 65 °C.

2.5. Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNAs isolated as described above were treated with DNase, and then reverse-transcribed with oligo $(dT)_{12-18}$ primer. After incubation with RNase H at 37 °C for 30 min, remaining primer was removed with SUPREC-02 (Takara, Japan). Real-time PCR assays were performed with GeneAmp 5700 Sequence Detection System (Applied Biosystems, USA) using SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 20 μl including cDNA template and appropriate primer pairs designed with the software Primer Express (Applied Biosystems). The amplification program consisted of 40 cycles of a first step at 95 °C for 15 s then a second step at 60 °C for 1 min. Quantification was based on the theory that the cycle threshold value, which is defined as the cycle number required to obtain a fluorescence signal above the background, correlates inversely with the log of the initial template concentration (Higuchi et al., 1992). The relative abundance of the targeted mRNAs was determined from a standard curve that was constructed from a set of dilution series of single-stranded cDNA. To normalize results, glyceraldehyde-3-phosphate dehydrogenase (DDBJ accession no. AB106523) was used as an internal standard. Primers for *ThCHS1* were: 5′-GGTTTGGTTTGGAACTTAATTTCAAT-3′ (forward primer) and 5′-TATTAACCATCAAAACATGCAACA-3′ (reverse primer). Primers for *ThCHS2* were: 5′-AGCGTGCCCTTGCCTAATAA-3′ (forward primer) and 5′-CCATTCCACACAAAGCGTGTT-3′ (reverse primer). Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: 5′-TCACTGTCAGGCTCGAGAAAGA-3′ (forward primer) and 5′-AGCCCAAAATGCCCTTAAGG–3′ (reverse primer).

2.6. Cloning of a CHS homologue

Amino acid sequences of CHS from plants were used to design degenerate primers for RT-PCR cloning of a new CHS homologue. The following degenerate primers were used (D = A or G or T, N = A or C or G or T, R = A or G, Y = C or T). 5′-ATGATGTAYCARCARGGNTG-3′ (forward primer), 5′-CTRTTCCARTCRCTDATNCC-3′ (reverse primer). cDNA was prepared from the 3′-UTRi transformant uI27 as described above. PCR reactions were cycled 30 times for 45 s at 94 °C, 45 s at 50 °C, and 1 min at 72 °C, respectively. Amplified PCR product was subcloned into pCR 2.1 (Invitrogen, USA) and sequenced. Based on the sequencing results, the full-length cDNA of the new CHS homologue was obtained with 3′-Full Race Core Set and 5′-Full RACE Core Set (Takara Bio Inc., Japan).

3. Results and discussion

3.1. Phenotypes of transformants

Eight lines of transformants expressing an inverted repeat sequence of a part of the *ThCHS1* CDS (CDSi transformants), and 18 lines of transformants expressing an inverted repeat sequence of a part of the *ThCHS1* 3′-UTR (3′-UTRi transformants) were obtained using *Agrobacterium*-mediated transformation. As a vector control, six lines of transformants transformed with pBI121 (Jefferson et al., 1987) were also obtained. All transgenic lines showed similar growth to wild type (WT) plants. There was no obvious unfavorable effects on plant growth in the CHS-suppressed transgenic lines (data not shown), although suppression of CHS activity would cease the production of flavonoids that show a wide range of functions such as UV-B protection and antimicrobial activities (Harborne and Williams, 2000).

All six lines transformed with pBI121 showed the same flower color as WT plants (cf. Fig. 3a). Seven of eight lines of CDSi transformants (designated as line Ix, where *x* represents arbitrary numbers) showed pure white flowers (Fig. 3b), and the remaining line (line I10) showed pale flower color (cf. Fig. 6a). The pale flower color of this transgenic line might be because of the introduction of mutations or disintegration of the inverted repeat sequence during transformation. In contrast, all 18 lines of 3′-UTRi transformants (designated as line uIx, where *x* represents arbitrary

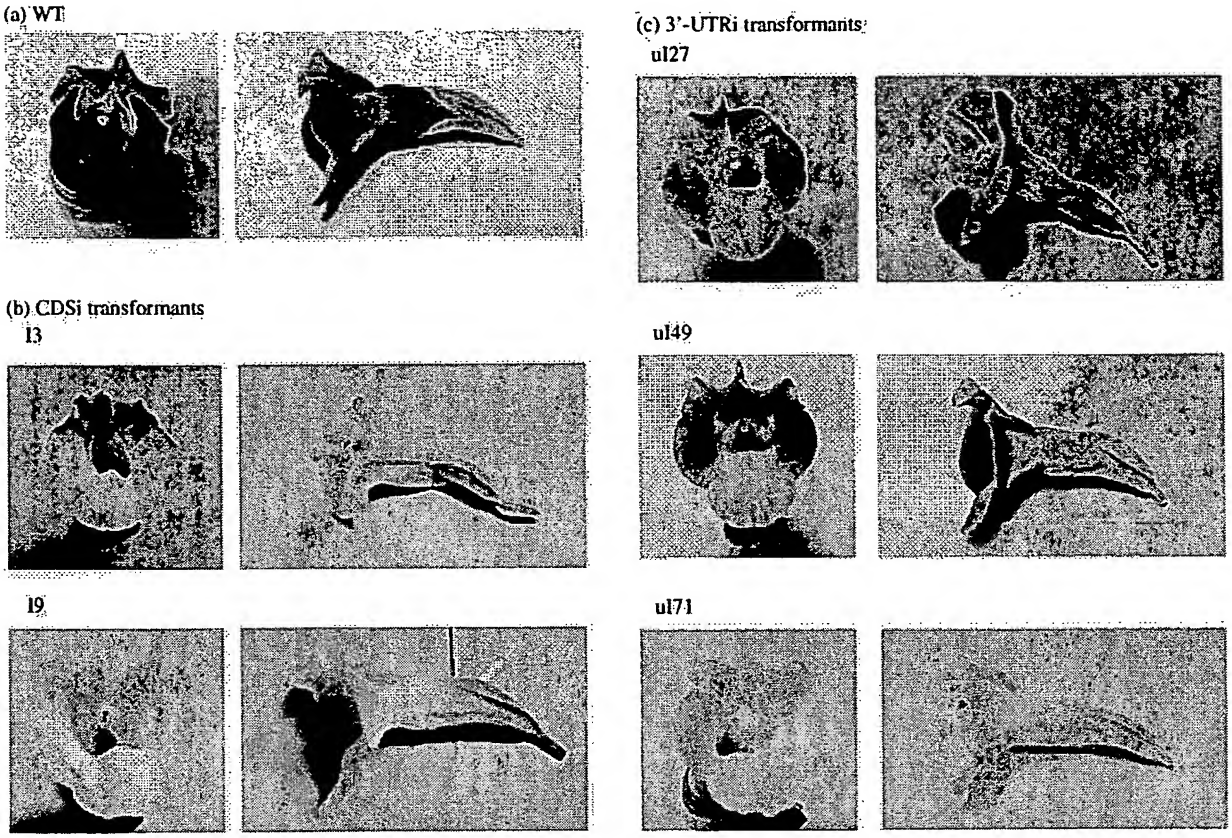

Fig. 3. Phenotypes of petals from wild type and transgenic plants. (a) Phenotypes of petals from wild type plants. WT, wild type. (b) Phenotypes of petals from CDSi transformants. Lines I3 and I9 are displayed as representatives. (c) Phenotypes of petals from 3′-UTRi transformants. Lines ul27, ul49, and ul71 are displayed as representatives.

numbers) showed various levels of pale flower colors (Fig. 3c).

On the whole, petals of CDSi lines showed pure white color, and those of 3′-UTRi lines showed pale colors. From these observations, we speculated molecular events occurred behind these phenotypic alterations as follows. Multiple CHS genes, sharing high sequence homology to each other, may contribute to flower color formation in *T. hybrida*. In the case of CDSi transformants showing pure white flowers, an exhaustive suppression of CHS genes was induced because of the their high sequence homology. On the other hand, only the target gene (*ThCHS1*) was specifically suppressed in 3′-UTRi transformants, and the remaining unsuppressed homologous genes contribute to the formation of pale flower colors. Variation of flower colors in 3′-UTRi transformants might be because of the difference of copy numbers of the inverted repeat sequence introduced in the genome.

3.2. Anthocyanin analysis

Over 80% of the total anthocyanins in the petals *T. hybrida* is malvidin-3-*O*-β-D-glucoside-5-*O*-(6-*O*-*p*-coumaroyl)-β-D-glucoside, and as a minor anthocyanin, peonidin-3-glucoside-5-(*p*-coumaroyl)-glucoside is also contained (Suzuki et al., 2000). In WT plants, two peaks derived from these two compounds were seen (black arrows in Fig. 4). Two other major peaks with maximum absorptions at ~250 and ~350 nm were also seen (white arrows in Fig. 4). These maximum absorptions are characteristic of flavonoids, suggesting that the two peaks were derived from colorless flavonoids. This was also confirmed

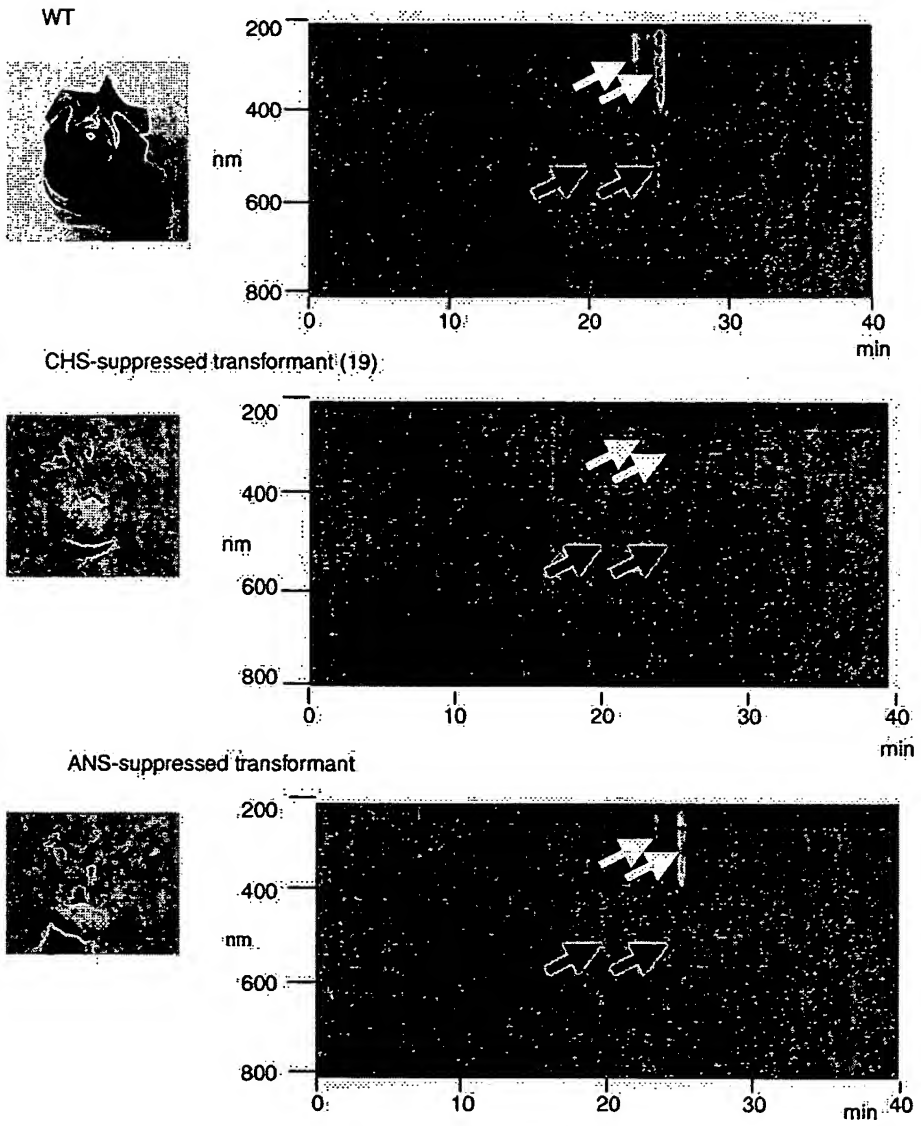

Fig. 4. HPLC chromatograms (photo-diode array detector profiles) of petal extracts prepared from wild type plants, CHS-suppressed plants (line I9), and ANS (anthocyanidin synthase)-suppressed plants. Flowers from these plants are also shown. Black and white arrows indicate peaks from anthocyanins and flavonoids, respectively.

with the HPLC profile from an ANS (anthocyanidin synthase: Fig. 1)-suppressed transformant (Mizutani et al., 2003) in which the two peaks from anthocyanins disappeared completely, while the two peaks indicated by white arrows in Fig. 4 remained unchanged (Fig. 4). This result also suggests that the two peaks were derived from flavonoids. To qualitatively examine the effect of RNAi on anthocyanin and flavonoid contents, a CHS-suppressed transformant was analyzed. The HPLC profile from a CDSi transformant (line I9) showed almost complete disappearance of all peaks because of the anthocyanins and flavonoids (Fig. 4), indicating that suppression of CHS blocked the biosynthetic pathways of both anthocyanins and flavonoids.

The quantity of anthocyanins was varied greatly between WT plants and transgenic plants (Fig. 5). WT plants contained approximately 1800–2000 ng

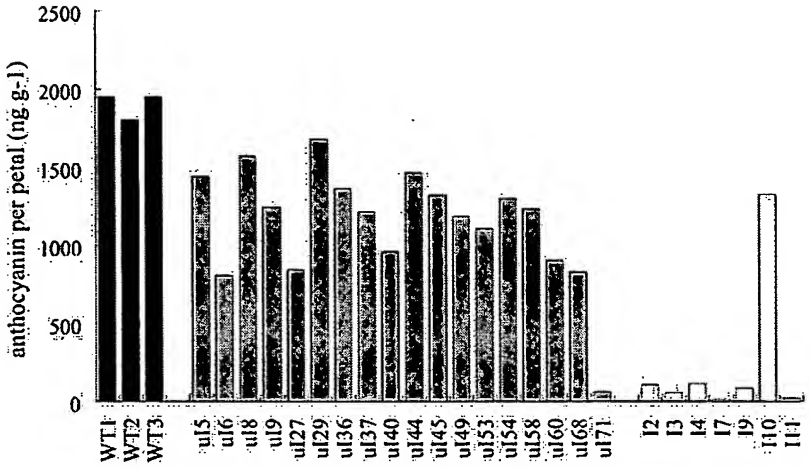

Fig. 5. Quantitative analysis of anthocyanins in petals from wild type plants and transgenic plants. WT, wild type plants; uIx, independent lines of 3′-UTRi transformants; Ix, independent lines of CDSi transformants (*x* represents arbitrary numbers).

of anthocyanins per 1 g of petals. In contrast, CDSi transformants contained only small amounts of anthocyanins (approximately 10–100 ng of anthocyanins per 1 g of petals) except for line I10 (~1300 ng of anthocyanins per 1 g of petals). Small content of anthocyanins coincided with flower color (pure white). 3′-UTRi transformants contained varying amounts of anthocyanins from approximately 60 to 1700 ng of anthocyanins per 1 g of petals. Variation of anthocyanin contents was consistent with paleness of flower color (cf. Figs. 3 and 6).

3.3. Expression analysis of CHS

Northern analysis was carried out to examine CHS gene expressions in petals of transgenic plants. A PCR product encoding a part of the CDS (black line in Fig. 2a) was used as a probe. As shown in Fig. 6a, no band was detected in CDSi transformants except for line I10, which showed pale flower colors. In contrast, pale but clear bands were observed in 3′-UTRi transformants, except for uI71, which showed petals of almost pure white (Fig. 6b). Thus, these results showed good correlation between intensity of bands and flower color.

To quantitatively examine expressions of CHS in transgenic plants, real-time quantitative RT-PCR was carried out for two CDSi transformants (I3 and I9), and four 3′-UTRi transformants (uI5, uI27, uI49, and uI71) using gene-specific primers designed based on the 3′-UTR sequence of *ThCHS1*. Expression levels of *ThCHS1* in CDSi transformants (I3 and I9) were approximately 14 and 12% of WT plants (Fig. 7a). Similarly, those in 3′-UTRi transformants (uI5, uI27, uI49, and uI71) were approximately 6, 11, 9, and 6% of WT plants (Fig. 7a). These results indicate that *ThCHS1* expression was strongly suppressed both in CDSi and 3′-UTRi transformants.

3.4. Southern blot analysis of CHS

To investigate whether homologous sequences of *ThCHS1* are present in the *T. hybrida* genome or not, Southern blot analysis was carried out using a PCR product encoding a part (black line in Fig. 2a) of the *ThCHS1* coding region (CDS) as a probe. The hybridization gave multiple bands in each case in which genomic DNA was digested with *Hin*dIII or *Eco*RI (Fig. 8). This result suggests that multiple copies of CHS homologues would be present in the genome of *T. hybrida* as well as *T. fournieri* (Aida et al., 2000).

3.5. Cloning and expression analysis of a new CHS homologue

To obtain a homologous gene of the known CHS gene (*ThCHS1*), degenerate PCR was performed using cDNA from a 3′-UTRi transformant (uI27) as a template according to the usual manners, and a new CHS homologue (designated as *ThCHS2* in this paper;

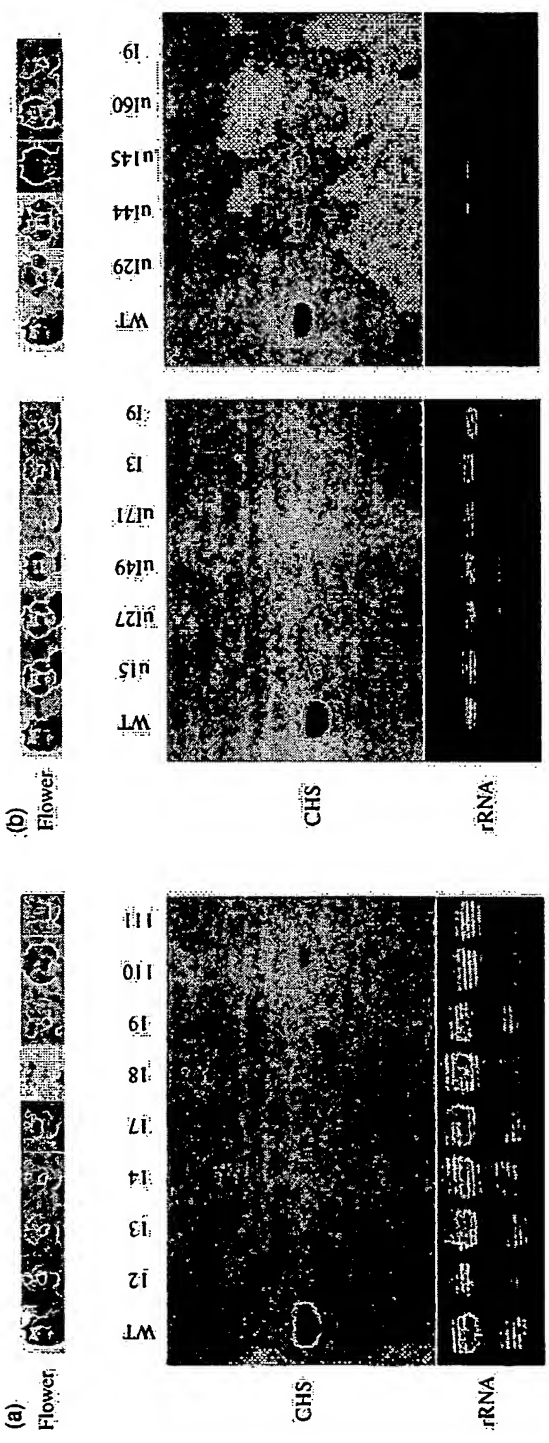

Fig. 6. Northern blot analysis of CHS in wild type (WT) and transgenic plants. Total RNA was extracted from buds of 1–7 days before flowering. Total RNA (8 μg) was separated on 1.5% agarose gels containing 2.2 M formaldehyde, and transferred to a nylon membrane. PCR product encoding a part of the CDS (black line in Fig. 2a) was used as a probe. (a) Northern blot analysis of CHS in WT and CDSi transformants. (b) Northern blot analysis of CHS in WT and 3′-UTRi transformants. For comparison, results of CDSi transformants (I3 and I9) are also shown. Samples shown in the left panel corresponds to those in Fig. 7a and b.

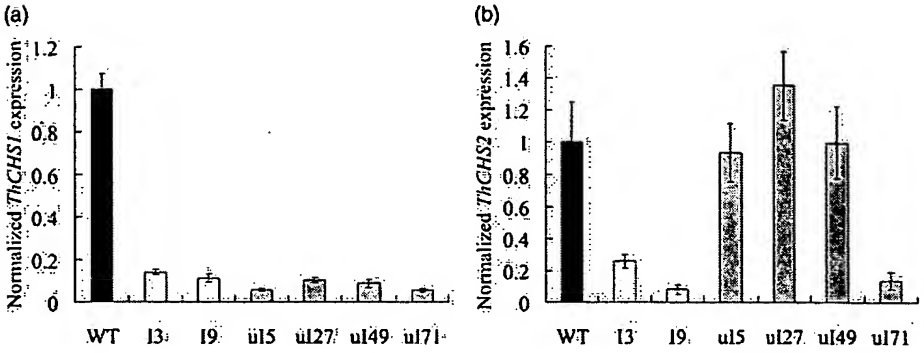

Fig. 7. Normalized expressions of CHS genes in transgenic plants determined by real-time RT-PCR. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal standard. *ThCHS1* and *ThCHS2* (a newly cloned CHS homologue) expressions in transgenic plants were normalized to that in wild type (WT) plants. Normalized expressions less than one indicate gene silencing. Data are the average of triplicate samples ± S.D. (standard deviation). (a) Normalized expressions of *ThCHS1*. (b) Normalized expressions of *ThCHS2*.

DDBJ accession no. AB106522) was cloned (Fig. 9). The CHS homologue has not been obtained from WT plants with the degenerate primers (data not shown). Alignment of nucleotide sequences of *ThCHS1* and *ThCHS2* revealed that they share high sequence identity (85%) in the CDS (boxed in Fig. 9), while they shared only 43% identity in the 3′-UTR. Sequence identity at the amino acid level was 92% (data not shown). It should be noted that four regions showing sequence identity longer than 20 bp were found in the part of CDS that was used as an RNAi target (crosses in Fig. 9). These regions might be the common target sites of RNAi in *ThCHS1* and *ThCHS2* when a part of CDS (black line in Fig. 2a) was used as an RNAi target.

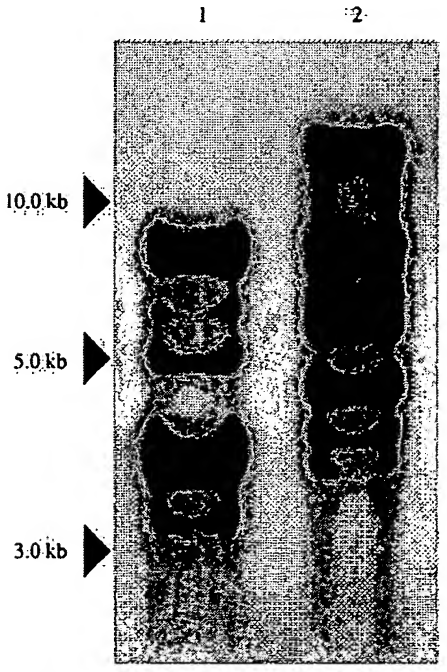

Fig. 8. Southern blot analysis of CHS in *T. hybrida*. DNA (10 μg) digested with *Hin*dIII (lane 1) or *Eco*RI (lane 2) was separated on 1% agarose gels and transferred to a nylon membrane. A part of the CDS (black line in Fig. 2a) of *ThCHS1* was used as a probe.

To quantitatively examine expression of *ThCHS2*, real-time RT-PCR with gene-specific primers was carried out. As shown in Fig. 7b, strong gene silencing was observed in CDSi transformants (I3 and I9) as well as in the case of *ThCHS1* (Fig. 7a). In contrast, no obvious suppression was seen in 3′-UTRi transformants (uI5, uI27, and uI49) except for uI71 (Fig. 7b). These results strongly suggest that exhaustive gene silencing against CHS homologues with high sequence identity was induced when the CDS of *ThCHS1* was targeted, while gene-specific RNAi against *ThCHS1* was induced when the 3′-UTR of *ThCHS1* was targeted. It is noteworthy that sufficient gene-specific silencing was achieved with a construct containing a short inverted repeat sequence encoding a part of the 3′-UTR (132 bp in length, gray arrow in Fig. 2a). Similar results were previously demonstrated by Ifuku et al. (2003) in *Nicotiana tabacum*, indicating that 3′-UTR is a potent target for gene-specific RNAi in plants. Our

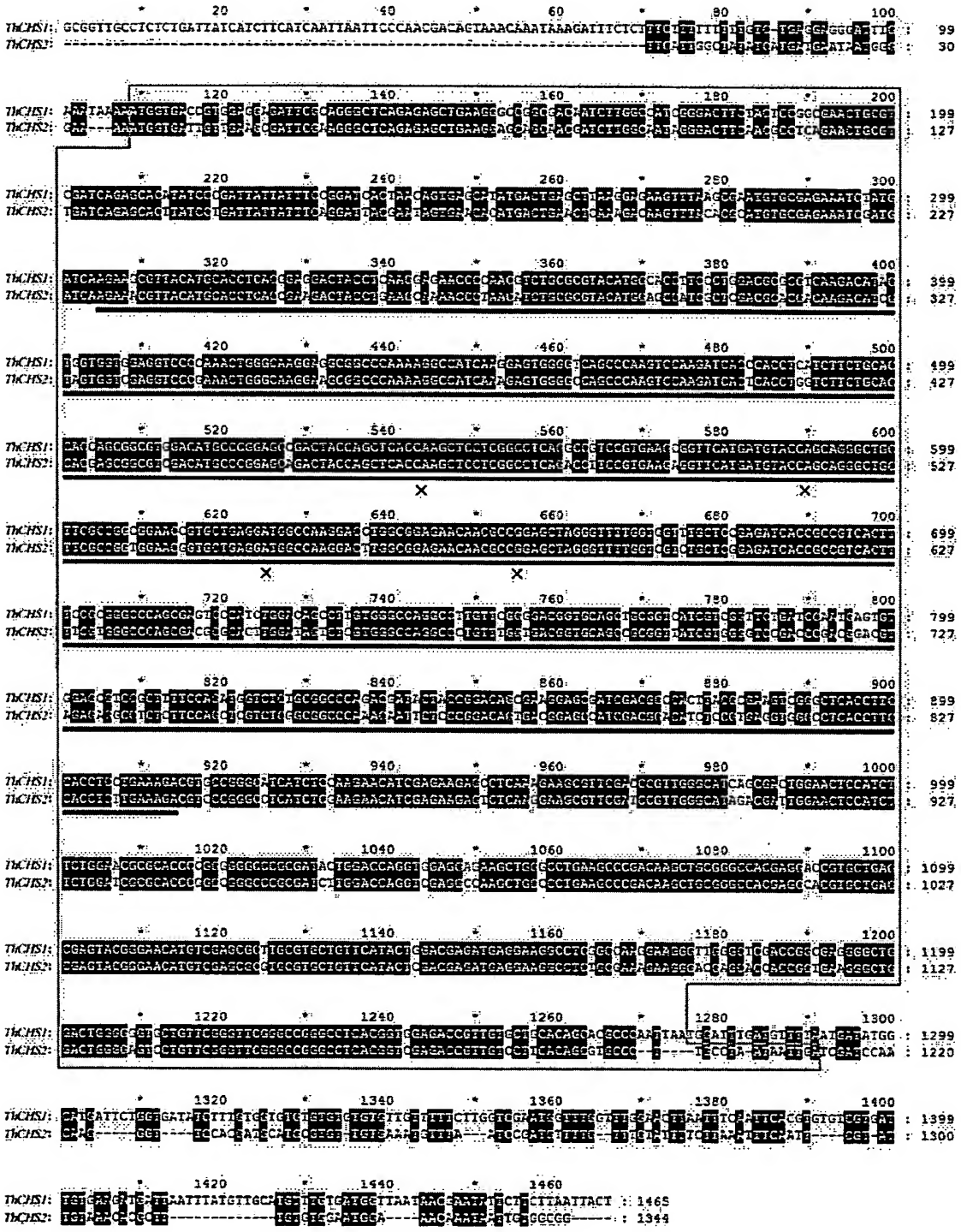

Fig. 9. Alignment of nucleotide sequences of *ThCHS1* and *ThCHS2*. Identical nucleotides are shown in black. Box indicates CDS, and the black line indicates the region used for the RNAi vector (CDSi) and Northern/Southern blot analysis (cf. Fig. 2a). Crosses indicate the regions showing sequence identity longer than 20 bp. These regions might induce RNAi against both *ThCHS1* and *ThCHS2* (for detail, see text).

results also indicate that exhaustive suppression of homologous genes can be achieved by using a region with high sequence identity (e.g. CDS) as an RNAi target. Comparison of data sets used to calculate expression levels of *ThCHS1* (Fig. 7a) and *ThCHS2* (Fig. 7b) revealed that expression levels of *ThCHS2* were about 1.5% of that of *ThCHS1* in WT plants. This result suggests that rarely expressed homologous gene(s) can be obtained by using gene-specific RNAi against abundant species. As shown in Fig. 7b, the expression of *ThCHS2* was strongly silenced in the 3′-UTRi transgenic line uI71. However, why only the line uI71 among 3′-UTRi transformants exhibited strong silencing for *ThCHS2* remains unclear.

In conclusion, we have succeeded in modulations of flower color of *T. hybrida* with RNAi technique. Furthermore, generation of different flower colors could be achieved by using different mRNA sites (CDS and 3′-UTR) as RNAi targets. This finding may be useful for creating novel (especially pale) flower colors. Our results demonstrate that RNAi is a powerful technique for flower color modulations of commercially important garden plants.

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